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The goal of this work is to study the regulation of the thymidine kinase (TK) gene in non-tumorigenic and tumorigenic human breast epithelial cells. Previous studies in our laboratory have shown that this gene is regulated during the cell cycle by both transcriptional and post-transcriptional mechanisms. The approach we are using in these studies is to examine the expression of hybrid genes in stably transfected breast cancer cells. Two hybrid genes have been constructed thus far: 1) TK-Luc, which contains the human TK promoter linked to a luciferase reporter gene, will be used to study transcriptional regulation; 2) CMV-TK*, which contains an epitope-tagged human TK cDNA expressed from a constitutive CMV promoter, will be used to study post-transcriptional regulation. These constructs have been transfected into non-tumorigenic (184B5) and tumorigenic (MCF-7) human breast epithelial cells. We are currently in the process of isolating and characterizing stably transfected cell lines. These transfectants will then be analyzed for regulation of the transfected gene(s). The results of these studies will identify the mechanism(s) that regulate cell cycle specific gene expression in normal breast epithelial cells, and establish whether these mechanisms are disrupted during the process of oncogenic transformation.

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Susan E. Conner 10/18/95
PI - Signature Date

# TABLE OF CONTENTS

# INTRODUCTION

Background: Abnormal control of cell proliferation is a hallmark of all types of cancer, including breast cancer. The regulation of proliferation occurs largely during the G1 phase of the cell cycle. One important control point that occurs late in G1 is the restriction point, or "R" (1). Once past "R", cells are committed to proceed to S phase and complete the remainder of the cell cycle. Several molecules seem to be involved in the regulation of progression through "R". These include the products of tumor suppressor genes such as the retinoblastoma protein (pRB), transcription factors such as E2F, protein kinases such as the cdk kinases, and regulatory molecules known as cyclins (2-9). The activities of these molecules are critical in maintaining normal cell growth controls, since mutations in a number of them, including pRB and cyclin D1, are associated with oncogenic transformation (10,11). While a role for molecules such as pRB, cyclins and cdk kinases in controlling progression from G1 to S is certain, relatively little is known about their targets of action. One attractive model is that they regulate genes that are induced at G1-S. Studying the regulation of such genes therefore provides an attractive approach to studying the activities of cell cycle regulators. For a number of years, my laboratory has studied the expression of one such gene, the human thymidine kinase (TK) gene. TK enzyme levels are tightly regulated by both the growth state and cell cycle position of the cell; rapidly dividing cells (including tumor cells) contain high levels of activity while resting cells contain low or undetectable levels (12). During the cell cycle, TK activity is low in G1, increases sharply as cells enter S phase, and remains elevated throughout S and G2 (13,14). The increase in TK activity at G1-S is regulated by many of the same events that regulate entry into S phase and DNA synthesis (15), and has therefore been used as a model to study the events that regulate passage through "R".

Experiments in my laboratory and others have shown that the increase in TK enzyme activity at G1-S is the result of both transcriptional and post-transcriptional controls. In terms of transcriptional control, we demonstrated that TK mRNA levels parallel enzyme activity during mitogenic stimulation of quiescent cells; that is, they are low in G0 and G1 and highly induced as cells enter S phase (16,17). The fact that this increase in TK mRNA is due (at least in part) to activation of the TK promoter has been shown in several ways. First, we demonstrated a 5-7 fold increase in the rate of transcription of the gene at G1-S in serum stimulated cells by nuclear run-on transcription assays (17). These results were later confirmed by other labs in additional cell lines (18). Second, we and other have shown that the human TK promoter confers G1-S regulation to linked heterologous genes (19-21). The sequences responsible for G1-S activation of the TK promoter have been mapped to a small region that contains binding sites for the cellular transcription factor E2F (19,22,23). E2F interacts with a number of cell cycle regulatory molecules including pRB, p107, cyclins A and E, and $p33^{cdk2}$ (7-9), and complexes containing several of these molecules have been reported to form with the TK promoter (24-26). Thus, TK transcription appears to be a direct target of cellular molecules that regulate entry into S phase, confirming our hypothesis that studying TK will give new insights into the mechanisms of action of these cell cycle regulators.

The 5-10 fold activation of the TK promoter at G1-S does not fully account for the much greater (up to 50-80 fold) increase in TK enzyme activity seen during the same interval, indicating that the gene is also regulated at a post-transcriptional level. In agreement with this,

several years ago we and others demonstrated that enzyme levels are regulated independently of mRNA levels when the TK cDNA is expressed from a heterologous promoter (21,27). In our experiments, in which the human TK cDNA was expressed from the SV40 early promoter in transfected Rat3 ($TK^-$) cells, mRNA levels were relatively constant during G1 and S phase, but TK enzyme levels were highly induced at G1-S. We therefore concluded that TK enzyme levels are regulated translationally or post-translationally at G1-S. We have recently demonstrated that the increase of TK protein at G1-S in serum stimulated Rat3 cells is due to a 10 fold change in the stability of TK protein between G1 and S phase, and not to a change in the rate of translation of TK mRNA (28). Translational regulation of TK has been reported in several other cases, however, including at the G1-S transition in cycling HeLa cells (14) and during serum starvation and differentiation of murine cell lines (29-31). In addition, TK protein stability has been shown to be regulated at mitosis in cycling cells (14,32). Thus, the specific mechanisms that regulate expression of the TK gene may vary according to the specific cell type and/or growth conditions being studied.

Purpose of Present Work: The overall hypothesis underlying this work is that molecules such as pRB, cyclins and cdk kinases exert their effects on the cell cycle (at least in part) by altering the expression of specific genes at G1-S. We plan to test this hypothesis by examining the role of cell cycle regulators in controlling the expression of TK, a model G1-S activated gene. The objectives of the research are threefold: 1) To characterize the mechanisms (transcriptional, translational and post-translational) that regulate TK expression in non-transformed human breast epithelial cells, 2) To identify the cell cycle regulatory molecules involved in control of the TK gene in these cells and 3) To determine if regulation of TK (and therefore the function of cell cycle regulatory molecules) is perturbed in transformed cells compared to normal cells. By comparing TK regulation in non-transformed and transformed cells, we hope to identify important changes in cell cycle regulation that are associated with oncogenic transformation. In the long term, this could lead to the development of novel screening strategies to identify tumors with high proliferative capacity (and therefore poor prognosis), so that these tumors could be treated aggressively.

Methods of Approach: The approach we are taking to address the objectives described above is to study the expression of hybrid genes in stably transfected human breast cell lines. To examine transcriptional and post-transcriptional regulation independently, we are using hybrid genes containing either (A) the human TK promoter linked to a heterologous (luciferase) reporter gene or (B) an epitope-tagged human TK cDNA construct expressed from an unregulated (CMV) promoter. These constructs have been or will be transfected into non-transformed and transformed human breast epithelial cells, and their regulation during the cell cycle will be examined.

# BODY

Our original Statement of Work contained the following 3 tasks to be carried out during the first year of this project. I will report our progress on each of these tasks below.

A) Construct hybrid genes to assay for transcriptional and post-transcriptional regulation.

B) Establish stably transfected cell lines expressing hybrid genes.

C) Study hybrid gene expression in stably transfected cell lines during mitogenic stimulation and during the cell cycle.

A) Construction of Hybrid Genes

We have constructed the following hybrid genes to be used in these studies.

1) TK-Luc: This gene contains a human TK promoter fragment containing sequences from -444 to +30 relative to the TK mRNA cap site linked to the firefly luciferase (luc) coding sequences. Previous experiments in our laboratory and others have demonstrated that this TK promoter fragment confers G1-S regulation to heterologous genes in transfected rodent cells. This gene will be used to study transcriptional regulation.

2) CMV-TK*: This gene contains an epitope tagged TK cDNA under the control of the CMV promoter. In our original proposal we planned to use a vector, pNHA, which contains sequences encoding a 10 amino acid epitope (HA1) from the influenza virus hemagglutinin protein, for these experiments. Because we were unable to obtain this vector from the person who constructed it, we have used an alternative method to produce an epitope tagged TK protein. Briefly, two primers were designed and used to amplify the human TK coding sequences as shown in Figure 1. The 5' primer (a 62 mer) contained a Bam H1 site followed by an AUG initiation codon, the coding sequences for the HA1 epitope, and 20 bases (in frame) from the TK cDNA. The 3' primer (a 30 mer) contained an Eco R1 site linked to TK cDNA sequences 102 bp downstream of the TK stop codon. These primers were used to amplify TK cDNA sequences from p5'TK cDNA, a functional TK cDNA clone that we prepared and have used previously (27). The PCR product was digested with Bam H1 and Eco R1, and subcloned into the vector pcDNA3 (In Vitrogen). This vector, which is shown in Figure 2, contains a CMV promoter for expression in mammalian cells. A clone containing the appropriate sized insert was isolated, and the entire insert was sequenced to insure that no mutations were introduced during the PCR amplification.

B) Establishment of Stably Transfected Cell Lines Expressing Hybrid Genes

Our initial transfections have been done using two human breast epithelial cell lines; 184B5, a non-tumorigenic cell line, and MCF-7, an estrogen dependent breast cancer cell line. We have not yet obtained stable transfectants in 184B5 cells using a $G418^r$ selectable marker, and are pursuing this objective by varying both transfection conditions and selectable markers.

We have obtained stable transfectants in MCF-7 cells, and report on our studies with these cell lines below.

i) Transfections using TK-Luc Reporter Gene: MCF-7 cells were co-transfected with TK-Luc DNA (5 μg/plate) and pSV2Neo (2 μg/plate) using lipofectin (Gibco/BRL). pSV2Neo contains a selectable G418$^r$ gene under the control of the SV40 early promoter. Stable transfectants were selected by growth in G418, and propagated as clonal cell lines. These cell lines were then assayed for expression of the luciferase reporter gene. None of the G418$^r$ cell lines tested thus far expressed appreciable levels of luciferase. We are therefore repeating these tranfections using a higher ratio of the unselected to the selected marker.

ii) Transfections using CMV-TK* Reporter Gene: MCF-7 cells were transfected with either the pCMV-TK* plasmid, or with the pcDNA3 vector, using lipofectin reagent. G418$^r$ colonies were selected, and propagated as clonal cell lines. Several of these cell lines were then assayed for expression of the transfected gene. The results of one such experiment are shown in Figure 3. Cytoplasmic extracts were prepared from two clonal cell lines (1C and 2G) containing pCMV-TK*, and one cell line (pcDNA3) containing the pcDNA3 vector. Twenty-five micrograms of protein from each extract were electrophoresed (in duplicate) on an SDS-polyacrylamide gel, blotted, and probed with antibodies to human TK (panel A) or to the influenza HA1 epitope (panel B). As shown in panel A, the pcDNA3 control cell line contains moderate levels of TK protein (which is expressed from the endogenous TK gene in MCF-7 cells). Both the 1C and 2G cell lines contain much higher levels of TK than the control, presumably because of the strength of the CMV promoter. As expected, the TK protein in cell lines 1C and 2G reacts with the anti-HA1 antibody (mAB 12CA5), while the endogenous TK protein in the control cell line does not. Thus, we have succeeded in expressing an epitope tagged TK protein in these cells. This protein is enzymatically active, as indicated by the fact that both 1C and 2G cell lines contain approximately 10 fold higher TK activity than does the control cell line (data not shown).

C) Studies of Hybrid Gene Expression in Stably Transfected Cell Lines during Mitogenic Stimulation and during the Cell Cycle.

We have initiated experiments to study the regulation of pCMV-TK* during the cell cycle in MCF-7 cells. To do this, we have established conditions to synchronize MCF-7 cells by estrogen depletion. MCF-7 cells require estrogen in order to proliferate and, in the absence of estrogen arrest in the G1 phase of the cell cycle. In our procedure, cells are plated in medium containing 5% FBS, which contains estrogen. On the following day, they are transferred to phenol-red-free medium containing 5% charcoal stripped serum (CSS) for 48-72 hrs. The charcoal stripping procedure removes estrogen from the medium. To assay for cell cycle position, nuclei were prepared, stained with propidium iodide, and analyzed for DNA content by flow cytometry. As shown in Table 1 below, approximately 92% of the cells are arrested in G1 after 2 days of estrogen depletion. In order to stimulate cells to re-enter the cell cycle and progress to S phase, fresh medium containing $10^{-9}$ M 17β-estradiol is added. As shown in the table, cells enter S phase beginning approximately 12 hrs after estradiol addition.

**Table 1: Cell Cycle Analysis of MCF-7 Cells in the Presence and Absence of Estrogen**

| Sample | % G0/G1 | %S | %G2/M |
|---|---|---|---|
| + E | 61.40 | 25.56 | 13.04 |
| - E | 92.76 | 4.74 | 2.50 |
| 2 hr, +E | 93.55 | 5.40 | 1.05 |
| 6 hr, +E | 92.53 | 4.53 | 2.94 |
| 12 hr, +E | 89.76 | 7.18 | 3.05 |
| 18 hr, +E | 49.56 | 46.99 | 3.45 |
| 24 hr, +E | 32.84 | 49.58 | 17.57 |

Since the transfected cell lines 1C and 2G are subclones of the original MCF-7 cells, we have analyzed their estrogen dependence. Both of these subclones maintain the estrogen dependence of the parental MCF-7 cells (data not shown). We are therefore in the process of evaluating whether pCMV-TK* is regulated during the cell cycle in these cells. If this construct is regulated, it will indicate that expression of the TK gene is regulated by translational and/or post-translational mechanisms in MCF-7 cells.

**CONCLUSIONS:**

In conclusion, during the first year of this grant we have constructed hybrid genes for the study of TK gene regulation in human breast epithelial cells. This includes one construct (TK-Luc) designed to study transcriptional regulation and a second (CMV-TK*) designed to study post-transcriptional regulation. We have had difficulty to date in obtaining stable transfectants in non-tumorigenic (184B5) cells. We are in the process of resolving this problem by trying different transfection conditions. We have obtained stable transfectants in the estrogen dependent MCF-7 breast cancer cell line, and have isolated and characterized several cell lines expressing an epitope-tagged human TK protein. Experiments to characterize the post-transcriptional regulation of TK during the cell cycle in these cells are currently underway. Co-transfections of TK-Luc and a $G418^r$ selectable marker have been carried out, and we are in the process of screening transfectants in order to identify cell lines that express the luciferase gene. These transfectants will be used to study transcriptional regulation of TK expression during the cell cycle.

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## APPENDIX: SUSAN E. CONRAD

## FIGURES 1-3

Figure 1

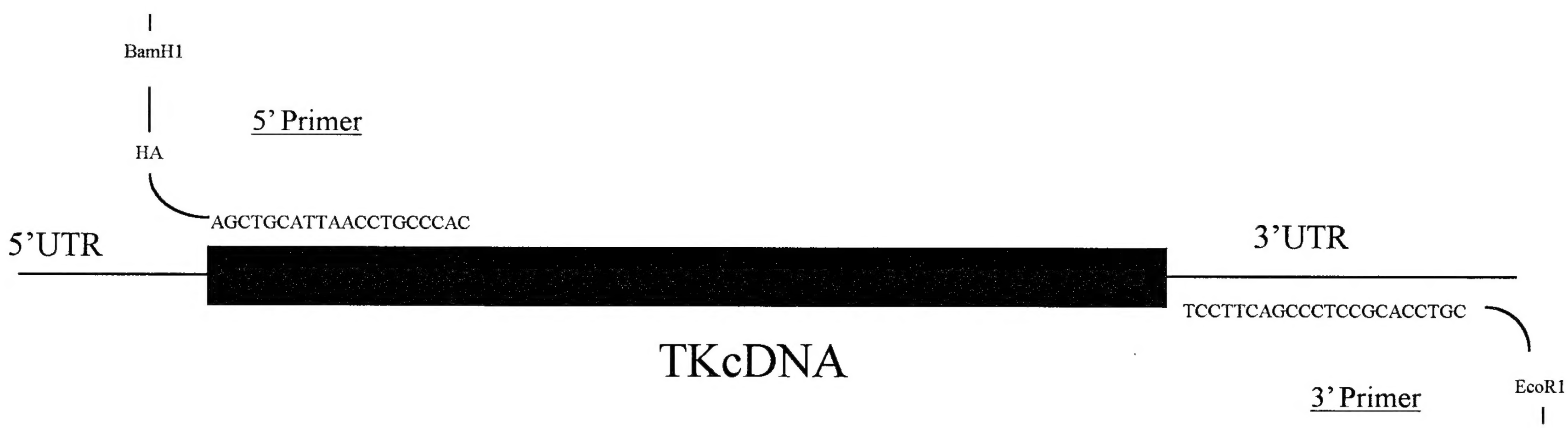


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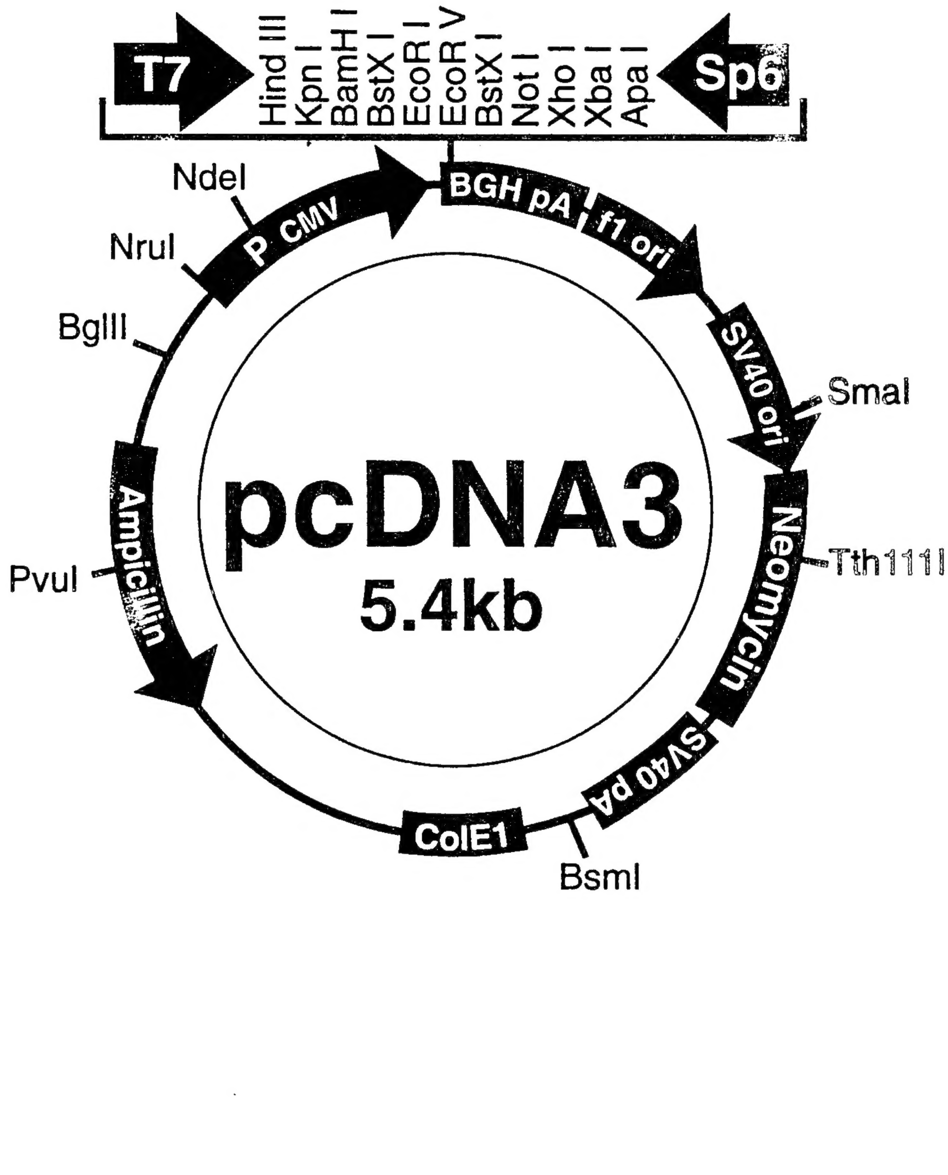
Figure 2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BamH I
BstX I
EcoR I
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Not I
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Sp6
NdeI
NruI
BglII
PvuI
P CMV
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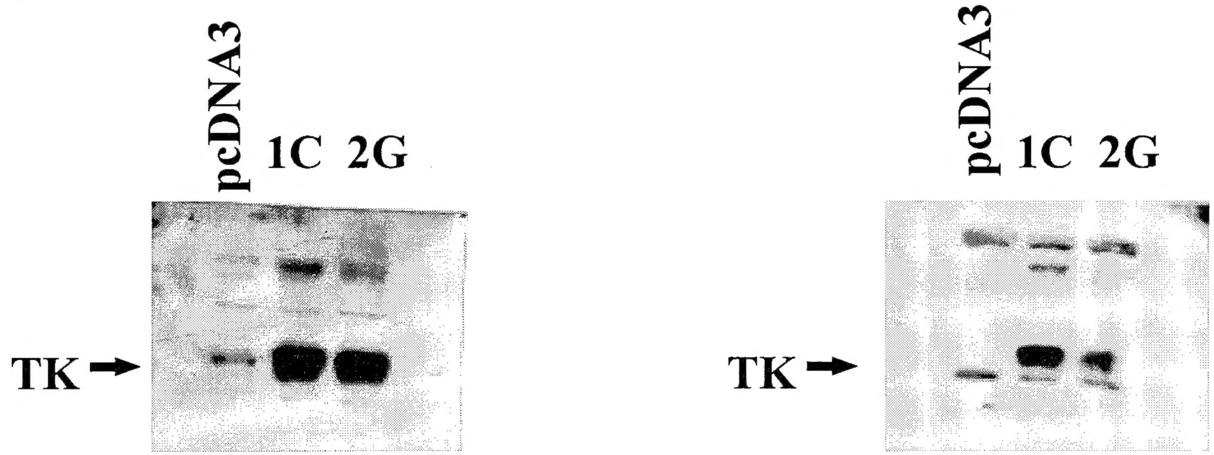
Figure 3




TK Probe

HA Probe